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Evaluation of resistance to fluoroquinolones and determination of mutations in *gyr*A and *par*C genes in *Escherichia coli* isolated from raw milk of dairy cows with coliform mastitis in Khorasan Razavi province, Iran

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ABSTRACT

The present study was performed to assess the resistance profile to fluoroquinolone and to determine mutations in gyrA and parC genes of *Escherichia coli* in bovine coliform mastitis. Fluoroquinolones (norfloxacin (NOR), ciprofloxacin (CIP), enrofloxacin (NFX), levofloxacin (LEV), and ofloxacin (OFL) were tested against *E. coli* isolates, isolated from bovine mastitis (100 milk samples) by disk diffusion method. To determine the extent of gyrA and parC mutations associated with fluoroquinolone resistance in *E. coli*, two isolates with the highest resistance to each fluoroquinolone were submitted for the amplification and sequencing of the quinolone resistance-determining regions (QRDRs) of gyrA and parC genes. The disk diffusion method indicated that *E. coli* isolates had the highest intermediate resistance to OFL (16.7%), followed by NFX and NOR (15%), while they had low resistance to CIP and LEV (3.33%). A few silent mutations in gyrA (in codons 91, 100, 111, 131, 132) and in parC (in codons 91, 157, 159) were detected in QRDRs, and mutations in nucleotides 65, 80, and 83 in gyrA, and 195, 209, 212 in parC were detected in the other isolate. These results showed an intermediate rate of resistance to fluoroquinolones in *E. coli* isolates from raw milk of cows with coliform mastitis

Keywords

Escherichia coli; fluoroquinolone resistance; gyrA gene; mastitis, parC gene

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Abbreviations

E. coli: *Escherichia coli*
FQ: Fluoroquinolone
QRDR: Quinolone resistance determining region
MC: MacConkey
EMB: Eosin Methylene Blue

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Introduction

Mastitis is considered one of the most important diseases in dairy animals that causes severe losses to the dairy industry [1]. The economic losses due to clinical mastitis include production loss, lower milk yield and value, treatment expenses, and loss of animal value [2]. Coliforms such as *Escherichia*, *Klebsiella spp.*, and *Enterobacter spp.* are the most common etiological agents causing clinical mastitis [3]. *Escherichia coli* is the most common species isolated from coliform mastitis which is a Gram-negative, non-spore-forming rod bacterium that belongs to the family *Enterobacteriaceae* [4, 5]. Clinical signs of *E. coli* mastitis include a wide range from a mild disease with only local inflammation changes in the mammary gland to severe with systemic signs, generally including high fever, increased pulse frequency, lack of appetite, decreased milk production, dehydration, rumen stasis, shock, and death [6, 7]. In cases of mild to moderate *E. coli* mastitis, the use of anti-inflammatory drugs and supportive treatments is recommended. In peracute or acute cases of *E. coli* mastitis, due to the potential risk of bacterial growth in the mammary gland, which in turn may lead to bacteremia, administration of broad-spectrum antimicrobials is recommended to reduce the number of bacteria [8].

The fluoroquinolones are broad-spectrum and bactericidal antibiotics. They are used against gram-positive and especially gram-negative bacteria such as members of the *Enterobacteriaceae* family [9, 10]. They block DNA synthesis by targeting bacterial DNA gyrase and topoisomerase IV, both of which are essential for bacterial DNA supercoiling as the replicating strands separate [11]. DNA gyrase and topoisomerase IV are tetrameric structures composed of two pairs of subunits. The four subunits of DNA gyrase include 2 monomers of A and 2 monomers of B, with the names GyrA and GyrB, respectively. The topoisomerase IV also has ParC and ParE subunits, which are encoded by parC and parE genes, respectively [12].

The major mechanisms of resistance to quinolone antibiotics include mutations that occur at the target drug sites, mutations that reduce drug accumulation, and plasmid-mediated quinolone resistance [13]. The most common mechanism that produces significant levels of clinical resistance to fluoroquinolones is an alteration in the target enzymes. These changes are caused by self-mutations occurring within the responsible genes. Resistance to fluoroquinolones is due to the substitution of amino acids in a certain region of GyrA or ParC subunits [13, 14]. The broad-spectrum activity of quinolones against various infections and the widespread use of these antibiotics, the abuse and unnecessary use of them, especially in developing countries, has accelerated the development of resistance mechanisms [15].

Fluoroquinolones are used in the treatment of infectious diseases, including coliform mastitis caused by *E. coli*. Since the drug resistance pattern has regional distribution, determination of this pattern of *E. coli* resistance can be used to determine the appropriate treatment regimen for clinical coliform mastitis [16]. This study aimed to determine the resistance pattern of *E. coli* isolated from cows with coliform mastitis to some fluoroquinolones and also to detect the mutations in QRDR of fluoroquinolone-resistance *E. coli* isolates.

Results

Identification of E. coli

In this study, 100 milk samples were subjected to isolation of *E. coli* by selective plating followed by streaking on the Eosin Methylene Blue (EMB) agar at 37 ˚C for 24 h. Typical colonies of *E. coli* were produced from 45 samples. These 45 presumptive *E. coli* isolates on EMB agar were confirmed by biochemical tests (Figure 1).

Susceptibility testing

The results of susceptibility study showed that less than 20% of *E. coli* isolates had intermediate resistance to each antibiotic (Figure 2). Intermediate resistance was 3.33% to ciprofloxacin and levofloxacin, 15% to enrofloxacin and norfloxacin, and 16.7% to ofloxacin (Table 1).

Identification of gyrA and parC mutations in clinical isolates of E. coli

Amplification of the QRDRs of gyrA and parC genes was performed by PCR (Figure 3). The results of the DNA sequencing of gyrA and those of parC were consistent and provided information from both standards for a region between nucleotides 247 to 840 (corresponding to codons 82 to 280) of gyrA and from nucleotides 167 to 539 (corresponding to codons 55 to 180) of the parC gene, respectively (Table 2, Figures 4, 5, 6, and 7). Accession numbers of *E. coli* isolates based on QRDRs of gyrA and parC genes deposited in the GenBank are as follows: SRX5988183, SRX5982112 for sample number 2968, and SRR17711097, SRR17711096 for sample number 3077 (Accession to cite for these SRA data: PRJNA547542).

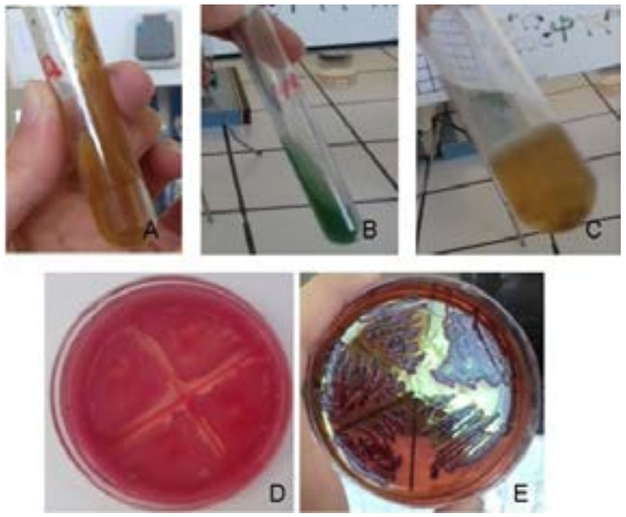

Figure 1.
Isolation and identification of *E. coli*. A) Triple Sugar Iron agar, acid/acid reaction with gas production and no H2S. B) Simmons citrate agar, the medium remained green. This is a negative result for citrate test. C) Sulfur Indole Motility (SIM) medium, *E. coli* is hydrogen sulfide negative, indole positive, and the cloudy appearance of the medium indicates that *E. coli* is motile. D) MacConkey agar, pink colonies. E) Eosin Methylene Blue agar, colonies of purple with black center and green metallic sheen.

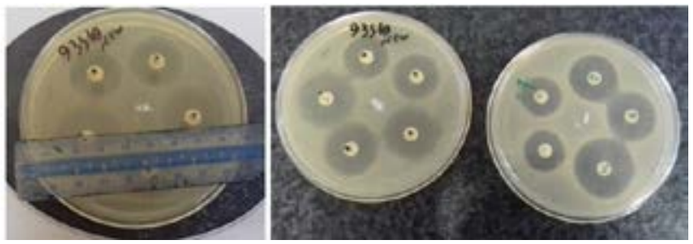

Figure 2.
The results of evaluation of resistance to fluoroquinolones with disk diffusion method. The scale is in mm.

Table 1.
Antibiotic resistance pattern of 60 *E. coli* isolates.

Antibiotic (µg)	No. of sensitive isolates	% sensitive isoates	% intermediate resistant isolates	% resistant isolates
Ofloxacin (5)	50	83.3	16.7	-
Enrofloxacin (5)	51	85	15	-
Norfloxacin (10)	51	85	15	-
Ciprofloxacin (5)	58	96.6	3.33	-
Levofloxacin (5)	58	96.6	3.33	-

Discussion

The focus of the current study was to assess the resistance of *E. coli* to some fluoroquinolones in bovine coliform mastitis and to generate the fluoroquinolones resistance profile of isolates. The level of resistance of *E. coli* isolates to enrofloxacin and norfloxacin was similar to the level of resistance to ofloxacin. This is due to the development of cross-resistance to one of the fluoroquinolones. In the present study, among all 60 *E. coli* isolates, less than 20% of isolates had intermediate resistance to fluoroquinolones. This is still a relatively low figure compared with other published studies, in which the proportion of resistant isolates has ranged from 23% to 63% [15, 17-19]. In the results from Su et al. (2016) *E. coli* isolates showed 4% resistance to ciprofloxacin and levofloxacin; similarly, *E. coli* isolates in the present study showed only 3.33% resistance to ciprofloxacin and levofloxacin whereas Metzger and Hogan (2013) found 12% of *E. coli* isolated from bovine milk samples were non-susceptible to ciprofloxacin [20, 21]. Among fluoroquinolones, enrofloxacin and norfloxacin resistance were found in nine (15%) *E. coli* isolates, and all other isolates

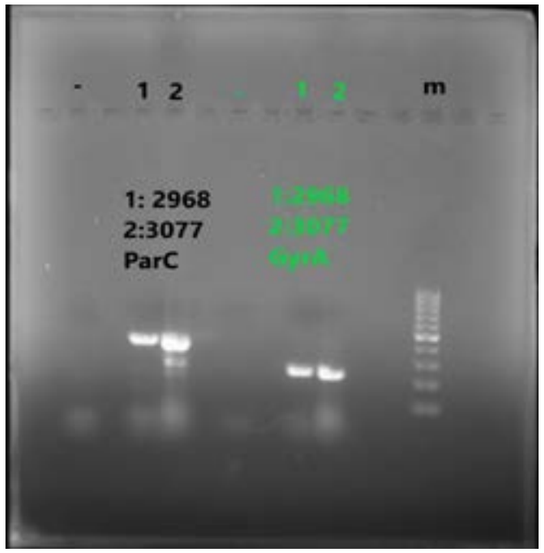

Figure 3.
PCR amplification of QRDR of gyrA and parC genes for *E. coli.*
Lane M: DNA marker; 100 bp plus. Lane 1, 2: test isolates. The expected product size of gyrA is 253 bp and the expected product size of parC is 434 bp.

Score	Expect	Identities	Gaps	Strand
261 bits(141)	2e-73	151/156(97%)	0/156(0%)	Plus/Minus

```
NC_000913.3 2227 CAGTTCATGGGCAATTTTCGCCAGACGGATTTCCGTATAACGCATTGCCGCCGCAGAGTC 2286
gyrA_2968   168  ........TT.............................................. 109

NC_000913.3 2287 GCCGTCGATAGAACCGAAGTTACCCTGACCGTCTACCAGCATATAACGCAGCGAGAATGG 2346
gyrA_2968   108  .........G.................................G.............. 49

NC_000913.3 2347 CTGCGCCATGCGGACGATCGTGTCATAGACCGCCGA 2382
gyrA_2968   48   .........A.......................... 13
```

Figure 4.
The result of alignment of the gyrA gene of sample number 2968. The red letters indicate nucleotide changes. Reference sequence is from nucleotide 2227 to 2382 from NC_000913.3:2336793-2339420 Escherichia coli str. K-12 substr. MG1655, complete genome.

Score	Expect	Identities	Gaps	Strand
678 bits(367)	0.0	385/393(98%)	3/393(0%)	Plus/Plus

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NC_000913.3 156 GGGCCTGAATGCCAGCGCCAAATTTAAAAAAT-CGGCCCGTACCGTCGGTGACGTACTGG 214
parC_2968   10  ...-...-.........................C.......................... 67

NC_000913.3 215 GTAAATACCATCCGCACGGCGATAGCGCCTGTTATGAAGCGATGGTCCTGATGGCGCAAC 274
parC_2968   68  ..........................................................G. 127

NC_000913.3 275 CGTTCTCTTACCGTTATCCGCTGGTTGATGGTCAGGGGAACTGGGGCGCGCCGGACGATC 334
parC_2968   128 ............................................................ 187

NC_000913.3 335 CGAAATCGTTCGCGGCAATGCGTTACACCGAATCCCGGTTGTCGAAATATTCCGAGCTGC 394
parC_2968   188 ............................................................ 247

NC_000913.3 395 TATTGAGCGAGCTGGGGCAGGGGACGGCTGACTGGGTGCCAAACTTCGACGGCACTTTGC 454
parC_2968   248 ............................................................ 307

NC_000913.3 455 AGGAGCCGAAAATGCTACCTGCCCGTCTGCCAAACATTTTGCTTAACGGCACCACCGGTA 514
parC_2968   308 ................G.....T..................................... 367

NC_000913.3 515 TTGCCGTCGGCATGGCGACCGATATTCCACCGC 547
parC_2968   368 ......................T.C....... 400
```

Figure 5.
The result of alignment the parC gene of sample number 2968. The red letters indicate nucleotide changes. Reference sequence is from nucleotide 156 to 547 from NC_000913.3:c3165973_3163715 Escherichia coli str. K_12 substr. MG1655, complete genome.

Score	Expect	Identities	Gaps	Strand
313 bits(169)	3e-89	204/221(92%)	1/221(0%)	Plus/Plus

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NC_000913.3 2204 GTCTCTTTTTCGAGATCGGCCATCAGTTCATGGGCAATTTTCGCCAGACGGATTTCCGTA 2263
gyrA_3077   5    ............................................................ 64

NC_000913.3 2264 TAACGCATTGCCGCCGCAGAGTCGCCGTCGATAGAACCGAAGTTACCCTGACCGTCTACC 2323
gyrA_3077   65   ..............................G.....................G..... 124

NC_000913.3 2324 AGCATATAACGCAGCGAGAATGGCTGCGCCATG-CGGACGATCGTGTCATAGACCGCCGA 2382
gyrA_3077   125  .....G.......A...A.........C..C.TA.....A....G......A......A. 184

NC_000913.3 2383 GTCACCATGGGGATGGTATTTACCGATTACGTCACCAACGA 2423
gyrA_3077   185  .A............................GAA.......... 225
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Figure 6.
The result of alignment the gyrA gene of sample number 3077. The red letters indicate nucleotide changes. Reference sequence is from nucleotide 2204 to 2423 from NC_000913.3:2336793-2339420 Escherichia coli str. K-12 substr. MG1655, complete genome

Score	Expect	Identities	Gaps	Strand
614 bits(332)	3e-180	359/371(97%)	5/371(1%)	Plus/Plus

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NC_000913.3 141 TGCGATGTCTGAACTGGGCCTGAATGCCAGCGCCAAATTTAAAAAATCGGCCCGTACCGT 200
parC_3077   26  ........-....-..........-C..CC.............................. 82

NC_000913.3 201 CGGTGACGTACTGGGTAAATACCATCCGCACGGCGATAGCGCCTGTTATGAAGCGATGGT 260
parC_3077   83  ............................................................ 142

NC_000913.3 261 CCTGATGGCGCAACCGTTCTCTTACCGTTATCCGCTGGTTGATGGTCAGGGGAACTGGGG 320
parC_3077   143 ............G............................................... 202

NC_000913.3 321 CGCGCCGGACGATCCGAAATCGTTCGCGGCAATGCGTTACACCGAATCCCGGTTGTCGAA 380
parC_3077   203 ...T.......................................................... 262

NC_000913.3 381 ATATTCCGAGCTGCTATTGAGCGAGCTGGGGCAGGGGACGGCTGACTGGGTGCCAAACTT 440
parC_3077   263 ............................................................ 322

NC_000913.3 441 CGACGGCACTTTGCAGGAGCCGAAAATGCTACCTGCCCGTCTGCCAAACATTTTGCTTAA 500
parC_3077   323 ..........................................................-..A.. 381

NC_000913.3 501 CGGCACCACCG 511
parC_3077   382 A.-........ 391
```

Figure 7.
The result of alignment the parC gene of sample number 3077. The red letters indicate nucleotide changes. Reference sequence is from nucleotide 141 to 511 from NC_000913.3:c3165973_3163715 Escherichia coli str. K_12 substr. MG1655, complete genome.

were susceptible to norfloxacin and enrofloxacin.

This is in general agreement with Malinowski et al. (2008) who found that 16.1% and 14.9% mastitis *E. coli* isolates from Poland were resistant to enrofloxacin and norfloxacin, respectively [22]. However, in a study in Bangladesh, no resistance to fluoroquinolones including ofloxacin, ciprofloxacin, and levofloxacin was reported in *E. coli* isolated from milk of mastitis cattle [23]. Persson et al. (2011) reported that there was no fluoroquinolone resistance in *E. coli* isolated from milk samples of cows with mastitis [24]. In another study by Persson and her colleagues in Sweden (2015), they reported that all isolates (n=57) of *E. coli* from dairy cows with acute clinical mastitis were susceptible to enrofloxacin [25]. Armanullah et al. (2018), studied the antibiotic resistance profile of *E. coli* isolates from bovine clinical mastitis and reported resistance to ciprofloxacin (16.67%), norfloxacin (8.33%), ofloxacin (8.33%), and intermediate resistance to norfloxacin (8.33%) that was somewhat similar to the finding of the present study [26].

Fluoroquinolone resistance of *E. coli* isolates from bovine mastitis has been studied by several authors and the results have varied, which may be due to different methods and breakpoints used to determine susceptibility. Resistance to fluoroquinolones is still uncommon among *E. coli* isolated from bovine mastitis. In comparison to other studies [15, 17-19, 27], the

results of this study showed a low level of resistance to fluoroquinolones, which may be due to the controlled use of these antibiotics. However, in the present study ciprofloxacin and levofloxacin were proved to be the best antibiotics to treat *E. coli* mastitis in cattle since they were highly effective.

In the present study, the *E. coli* isolates did not have resistance to fluoroquinolones and the rate of intermediate resistance to fluoroquinolones was very low. It is generally accepted that gyrA mutations play a major role in the development of fluoroquinolone resistance in *E. coli,* while the mutations in the parC gene are additionally associated with resistance [28]. To analyze the correlation between genetic characterization and resistance phenotype, two isolates with the most resistance to each fluoroquinolone were submitted to amplification and sequencing of the QRDR in gyrA and parC genes. There were two silent mutations in the gyrA gene at wobble position in codons 91 and 100; similarly, *E. coli* isolates in the Heisig study showed silent mutations in codons 91 and 100 [28]. Mutation at codons 83 and 87 was found to be the most common gyrA mutations of *E. coli* in several studies, and in the present study, there was a mutation in codon 83 of gyrA in sample number 3077 [29-32]. In addition, we found a silent mutation in codon 91 in the parC gene. Similarly, *E. coli* isolates in the Heisig study showed silent mutation only in codon 91, whereas the most common mutations in parC were reported at codons 80, 84, and 87 [19, 28, 31, 32].

In conclusion, the current investigation showed that most *E. coli* isolates isolated from raw milk of cows with coliform mastitis in Khorasan Razavi province were sensitive to fluoroquinolones and some *E. coli* isolates had intermediate resistance to fluoroquinolones. In gyrA and parC genes of *E. coli* isolates with the most intermediate resistance to studied fluoroquinolones, there were silent mutations and mutations. There is some evidence that silent mutations can especially affect the regulation of transcription [33-35].

Materials & Methods

Sample collection

A total of one hundred (100) milk samples were examined in this study. Samples were collected from the milk of dairy cattle with clinical mastitis of three dairy farms in Mashhad (Khorasan Razavi province, Iran). Fifteen isolates of *E. coli* were obtained from "Bacterial Collection of the Mastitis Laboratory", Veterinary Teaching Hospital, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad.

Isolation of E. coli

Milk samples were cultured on MacConkey agar media (Merk, Darmstadt, Germany) and were incubated at 37 ˚C for 24 h. Suspected E. coli lactose-fermenting colonies (pink colonies) were used for culture on the Eosin methylene blue (EMB) agar (Merk, Darmstadt, Germany). The appearance of the colonies of purple with black center and green metallic sheen were considered positive for *E. coli* on EMB agar and were selected for further studies. The colonies of presumptive *E. coli* on EMB agar were confirmed by standard biochemical tests, including triple sugar iron agar, Simmons citrate agar, and motility assay.

Antibiotic susceptibility study

Antibiotic susceptibility testing was carried out with equivalence of 0.5 McFarland turbidity standard by agar disk diffusion method on Mueller-Hinton agar (Himedia, Mumbai, India) plates following the Clinical and Laboratory Standards Institute [36]. All *E. coli* isolates were subjected to an antibiotic susceptibility test. The antimicrobial disks (Padtan Teb, Tehran, Iran) used in the experiment included 5 µg ciprofloxacin, 10 µg norfloxacin, 5 µg levofloxacin, 5 µg ofloxacin, and 5 µg enrofloxacin. The antibiotic disks were placed on Mueller-Hinton agar culture plate. The plates were incubated for 18-24 h at 37 ˚C. The size of the zone of inhibition was recorded and resistance zone diameter breakpoints adopted for these antimicrobials were the following: ≤ 15 mm for ciprofloxacin, ≤ 12 mm for norfloxacin, ≤ 13 mm for levofloxacin, ≤ 12 mm for ofloxacin, and ≤ 14 mm for enrofloxacin.

Table 2.
Mutations in genes *gyrA* and *parC*.

E. coli isolate	gyrA mutation			parC mutation		
	Codon position	Nucleotide exchange	Amino acid exchange	Codon position	Nucleotide exchange	Amino acid exchange
2968	91	CGT → CGC	ginine [a]	91	CAG → CAA	Glutamine [a]
	100	TAC → TAT	Tyrosine [a]	157	CTG → CTA	Leucine [a]
	111	TCC → TCT	Serine [a]	159	GCT → GCC	Alanine [a]
	131	GCA → GCC	Alanine [a]			
	132	AAT → CAT	Asparagine → Histidine			
3077	65	AAT → CAT	Asparagine → Histidine	195	GGT → GTT	Glycine → Valine
	80	GCA → TCA	Alanine → Serine	209	GTG → TTA	Valine → Leucine
	83	AGG → GGG	Arginine → Glycine	212	GGC → CAC	Glycine → Histidine

[a] Silent mutation

DNA extraction

E. coli isolates were grown overnight in Nutrient agar (Merk, Darmstadt, Germany) at 37 ˚C. One colony was suspended in 250 µL of sterile distilled water. After boiling the suspension for 15 min, followed by freezing and subsequent centrifugation at 14000 rpm for 15 min, the cell debris was pelleted and the supernatant was used as a template for the amplification reaction.
[37].

Amplification of quinolone resistance determining regions (QRDRs)

Polymerase chain reaction (PCR) was used to amplify QRDR of gyrA and parC for mutation detection. The list of primers that were used for amplification of gyrA and parC genes is shown in Table 3. The PCR amplification was performed in a total reaction volume of 25 µL. The reaction mixture contained 12.5 µL of 2x master mixtures (CinnaGen, Tehran, Iran), 1 µL of each forward and reverse primer (10 pmol/µL), 8.5 µL of deionized water and 2 µL of DNA template. The PCR program included initial denaturation at 94 ˚C for 5 min followed by 30 cycles of denaturation (94 ˚C for 1 min), annealing (55 ˚C for gyrA and 56 ˚C for parC for 1 min), and extension (72 ˚C for 1 min) with a final extension at 72 ˚C for 5 min. For amplification of DNA, the PCR was performed with a thermocycler (Techne, Chelmsford, UK). The PCR products were run on a 1% agarose gel in TAE buffer at 100 V for 45 min. After electrophoresis (Padideh Nojen Pars, Mashhad, Iran) in the agarose gel and staining with the green viewer (Sinaclone, Tehran, Iran), they were observed and documented under gel documentation system (Kimiagene, Mashhad, Iran). A 100 bp plus DNA ladder was used to determine the molecular size of the PCR products. Primers used in the study were custom synthesized from Macrogen Inc. (South Korea).

Sequencing and Alignment

The PCR product of gyrA and parC genes with forward and reverse primers sent for sequencing to Microsynth (Switzerland). DNA sequences were analysed using Chromas software. DNA sequence data were compared to data in the GenBank database using the BLAST algorithm available at the National Center for Biotechnology Information (NCBI) website (www.ncbi.nlm.nih.gov).

Table 3.
The primers were used for amplification of *gyrA* and *parC*

Primer name	Primer direction [a]	Sequence (5' to 3')	Product size (bp)	Annealing temperature (°C)	Reference
gyrA4	F	TCGTTGGTGACGTAATCGGT	253	55	31
gyrA5	R	TCCGTGCCGTCATAGTTATC	253	55	31
parC1	F	AACCTGTTCAGCGCCGCATT	434	56	31
parC2	R	ATGCGGTGGAATATCGGTCG	434	56	31

[a] F, forward; R, reverse

Authors' Contributions

B.F., A.J., and B.KH. conceived and planned the experiments. M.M. carried out the experiments. B.F., A.J. and B.KH. planned and carried out the simulations. M.M., K.L., and B.KH. contributed to sample preparation. B.F., A.J., and B.KH. contributed to the interpretation of the results. B.F. took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis, and the manuscript.

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Conflict of interest

The authors declare that they have no competing interests.

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